Biosynthetic Studies of Amphidinolide J: Explanation of the Generation of the Unusual Odd-numbered Macrocyclic Lactone

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The biosynthetic origin of amphidinolide J **1** is investigated by means of feeding experiments of labeled [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$] sodium acetate with cultures of the dinoflagellate *Amphidinium* sp. which reveal that amphidinolide J **1** is generated through non-successive mixed polyketides, this may explain the generation of the unusual odd-numbered macrocyclic lactone ring.

Macrolide natural products generally possess even-numbered macrocyclic lactone rings.[1] However, several odd-numbered macrolides were recently isolated from the laboratory-cultured marine dinoflagellates *Amphidinium* sp.,[2,3] which are found in Okinawan marine flatworms, *Amphiscolops* sp. These macrolides, amphidinolides, have other unique structural features: (*a*) they have a variety of novel backbone-skeletons, isolated from one genus of microalga; (*b*) all amphidinolides contain at least one *exo*-methylene unit, and (*c*) vicinally located one-carbon branches (methyl or *exo*-methylene) are present in amphidinolides B–D, F–H, J–M and amphidinin A.[4] The generation of odd-numbered macrocyclic lactone rings (amphidinolides C, E–G and J–M) as well as the structural feature (*c*), cannot be accounted for by the normal polyketide biosynthesis. Although extensive biosynthetic studies based on NMR data of ^{13}C-labelled samples have been carried out for polyketide antibiotics produced by terrestrial microorganisms,[5] few studies have been reported for those from marine organisms.[6–11] We now describe the studies on the biosynthesis of amphidinolide J **1**,[12] currently the most abundant macrolide in *Amphidinium* sp. (strain Y-5), on the basis of stable isotope incorporation experiments. The backbone of amphidinolide J **1** was shown to be derived from non-successive mixed polyketide chains which may explain the generation of unusual odd-numbered (15-membered) lactone rings.

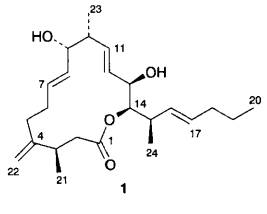
The dinoflagellate *Amphidinium* sp. (strain Y-5) was cultured in glass bottles (3 l) containing a nutrient-enriched sea water medium as previously described,[2a] and feeding experiments were carried out with [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$] sodium acetate and [*methyl*-^{13}C]-L-methionine. These ^{13}C-labelled precursors were fed to the algae (610 μmol dm^{-3}, $MeCO_2Na$; 93 μmol dm^{-3} methionine) in one portion 10-12 d after inoculation, then 2 days later the culture was harvested.† The extract of the harvested cells was purified by improved procedures‡ to afford ^{13}C-labelled amphidinolide J **1** (0.5-1 mg from 80-100 l of culture). Assignments of the ^{13}C NMR signals of **1** in C_6D_6 solution were fully established by HMQC and HMBC spectra and are presented in Table 1. The ^{13}C NMR spectrum of amphidinolide J **1** derived from [1-^{13}C] sodium acetate showed significant enrichment of nine carbons (C-1, C-4, C-6, C-8, C-10, C-13, C-15, C-17 and C-19), while **1** derived from [2-^{13}C] sodium acetate showed enrichment of 15 carbons (C-2, C-3, C-5, C-7, C-9, C-11, C-12, C-14, C-16, C-18 and C-20–24). The ratios of the signal intensities over those of nonlabelled **1** are also described in Table 1. Thus, all 24 carbons contained in amphidinolide J **1** were shown to be derived from acetates. The ^{13}C NMR spectrum of **1** obtained from the feeding experiment with [*methyl*-^{13}C]-L-methionine did not show appreciable enrichment of any carbon.§ The ^{13}C–^{13}C coupling constants ($^1J_{CC}$) of **1** labelled with [1,2-$^{13}C_2$] acetate (Table 1) indicated that the C_2 units for C-1/C-2, C-4/C-5, C-6/C-7, C-8/C-9, C-10/C-11, C-13/C-14, C-15/C-16, C-17/C-18 and C-19/C-20 originate from the same acetates.

The labelling patterns of amphidinolide J **1** shown by the feeding experiments were quite unusual, Fig. 1. Significantly, the C-3 and C-12 of **1** were derived from the methyl carbons of acetates, the carboxyl carbons of which were lost. Thus, the

Table 1 Isotope incorporation results based on the ^{13}C NMR data of amphidinolide J **1**[a]

Position	δ_C	Intensity ratio (labelled/unlabelled)[b] [1-^{13}C]-acetate	[2-^{13}C]-acetate	Assignment ● or ▲[c]	J_{CC}/Hz [1,2-$^{13}C_2$]-acetate
1	171.6	1.41	1	●	57.8
2	39.9	1.01	1.72	▲	57.8
3	34.6	0.88	1.59	▲	—
4	151.9	1.34	0.97	●	42.5
5	36.1	1.05	2.02	▲	41.4
6	29.7	1.68	1.36	●	43.6
7	130.8	0.87	1.88	▲	43.6
8	136.5	2.10	1.10	●	49.0
9	78.8	0.95	1.66	▲	48.0
10	45.7	1.51	0.99	●	43.6
11	133.5	1.03	2.09	▲	43.6
12	132.6	0.87	1.51	▲	—
13	72.6	1.46	1.05	●	42.5
14	79.9	1.15	2.33	▲	41.4
15	39.5	1.52	1.16	●	42.5
16	133.6	0.76	1.61	▲	43.6
17	131.5	1.53	0.95	●	42.5
18	35.3	0.94	2.50	▲	42.5
19	23.4	1.58	1.01	●	34.9
20	14.2	1	1.98	▲	34.9
21	22.2	0.96	1.81	▲	—
22	108.7	1.12	1.99	▲	—
23	19.0	1.03	1.78	▲	—
24	17.5	1.14	2.21	▲	—

[a] The ^{13}C NMR spectra were recorded in C_6D_6 solution on a Bruker ARX500 spectrometer at 125 MHz with sweep width of 35700 Hz using Bruker's pulse program 'zgpg30'. Numbers of scans were *ca.* 13000 and 25728, for the samples from feedings of mono- and double-^{13}C labelled precursors, respectively. [b] Intensity of each peak in the labelled **1** divided by that of the corresponding signal in the unlabelled **1**, normalized to give a ratio of 1 for an unenriched peak (C-20 for [1-^{13}C]acetate labelling and C-1 for [2-^{13}C]-acetate labelling). [c] ● denotes carbon derived from C-1 of acetate, while ▲ indicates carbon derived from C-2 of acetate.



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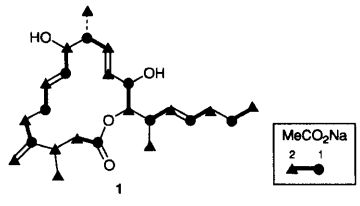

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Fig. 1 Labelling patterns of amphidinolide J **1** resulting from feeding experiments with ^{13}C-labelled precursors

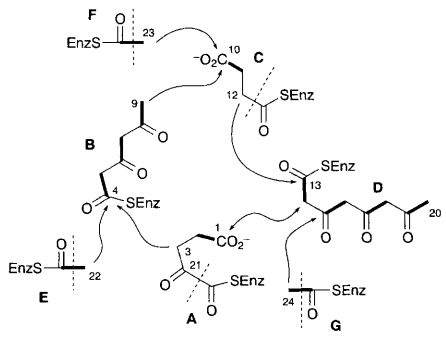

Fig. 2 Possible biosynthetic building blocks of amphidinolide J **1**

15-membered lactone ring was not constructed from a consecutive polyketide chain. This finding seems to justify that the lactone ring size of **1** does not have to be even. The irregular labeling pattern of **1** could be interpreted by assuming that the backbone carbons of **1** were biosynthetically derived from the precursors depicted in Fig. 2.¶ Units **B** (C-4 to C-9) and **D** (C-13 to C-20) are likely to be classical polyketides derived as a result of the condensation of three and four acetate units, respectively. Unit **A** (C-1/C-2/C-3/C-21) contains the '●–▲–▲–▲' group and may come from a dicarboxylic acid, *e.g.* α-ketoglutarate, after passage of acetate through the TCA cycle, which has been observed in the biosynthesis of brevetoxin B (C-6/C-7/C-8/C-9)[7] and okadaic acid (C-8/C-9/C-10/C-43).[8] Unit **C** (C-10/C-11/C-12) labelled as '●–▲–▲' may be derived from succinate,‖ corresponding to the six units of brevetoxin **B** (*e.g.* C-10/C-11/C-12).[7] Units **E**, **F** and **G** (C-22, C-23 and C-24) are one-carbon branches (an *exo*-methylene and two secondary methyls), and they were demonstrated to be derived from the C-2 of acetates and attached to carbons in a linear chain derived from the C-1 of acetates (C-4, C-10 and C-15, respectively). One-carbon branching of this type is unusual in polyketide biosynthesis and has been previously reported for only a few systems.[7,8,10a,13] Another one-carbon branch of C-21 also came from the C-2 of acetate.†† However, the condensation of this carbon into the linear chain occurred at the carbon (C-3) derived from the C-2 of the acetate; thus, the participation of a dicarboxylic acid precursor was proposed for this moiety (*vide supra*). How the vicinal locations of one-carbon branches[14] are brought about in amphidinolides is still an interesting question, the present results suggest that the two vicinal one-carbon branches (C-21 and C-22) of **1** were both derived from the C-2 of acetate but attached to the linear chain through different processes. It should be noted that the oxymethines at C-9 and C-14 of **1** are derived from the C-2 of acetate, and the origins of the oxygen atoms are unknown.

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Footnotes

† When the culture was harvested 4 days after feeding labelled sodium acetate to the algae, the ^{13}C NMR spectrum of isolated **1** showed that all carbon atoms of **1** were enriched and almost all signals were observed with double satellite signals due to vicinal ^{13}C–^{13}C couplings. This phenomenon was probably observed because C-1 of the acetate was cleaved via decarboxylation during passage through the TCA cycle and the released $^{13}CO_2$ was reincorporated during photosynthesis to give randomly labelled acetates, which led to all-carbon enriched **1**.

‡ The MeOH–toluene (3 : 1) extract of the harvested cells (*ca.* 18 g wet mass/100 l of culture) was partitioned between toluene and 1 mol dm^{-3} NaCl. The toluene-soluble fraction was subjected to successive flash chromatography on ODS (YMC-GEL ODS 60, I-40/60, 20 x 60 mm; 20% MeOH) and silica gel [Wakogel C-300, 20 x 400 mm; hexane–acetone (85:15)], followed by final purification by HPLC (Develosil ODS-5, 5 μm, 10 x 250 mm; 30% H_2O) to give amphidinolide J **1** (t_R 18.7 min) in each feeding experiment.

§ At least under our conditions of the feeding experiments (the spectrum was shown in the supplementary data for the referees' information).

¶ The biosynthetic building blocks depicted in Fig. 2 are only one possibility. There may be other several ways in which the observed labelling pattern could be derived biosynthetically.

‖ Unusual 1,4-polyketides (amphidinoketides) were recently isolated from *Amphidinium* sp. and a possible biosynthetic pathway involving the condensation of succinates was proposed.[15]

††In the ^{13}C NMR spectrum of **1** derived from the [1,2-$^{13}C_2$]-acetate experiment there were small satellites associated with the carbons of C-21–24. We are grateful to a referee, who indicated this question and provided the following proposal. It is statistically unlikely that they are couplings to carbons from other acetate units and more likely that there is some intact incorporation of the acetate units possibly in a different orientation. It is intriguing and possibly indicates the operation of two different pathways (one major and one minor) from acetate, generating a common intermediate.

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